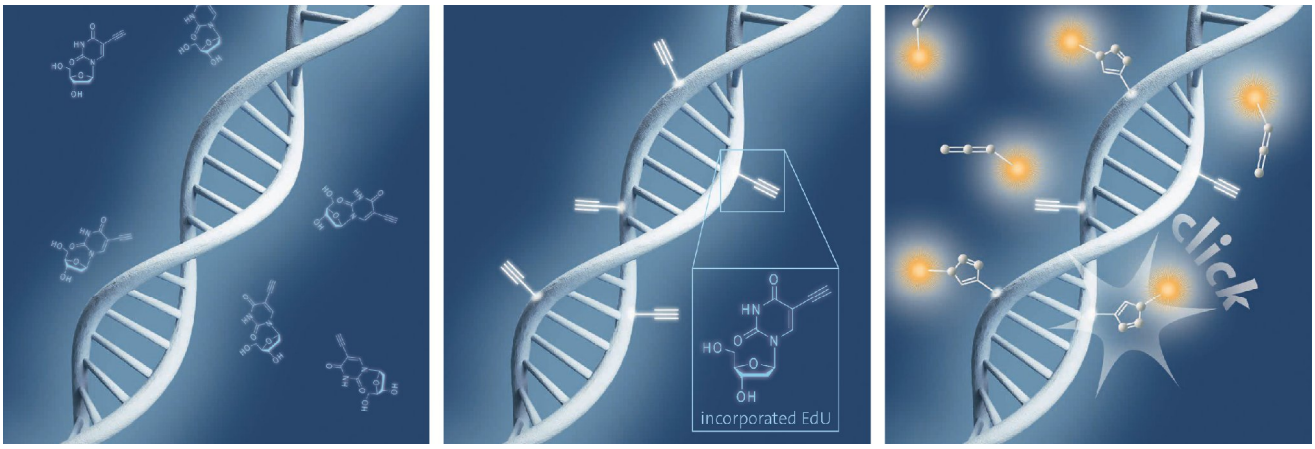
USER MANUAL

EdU Click HTS

ROTI®kit for High Throughput Screening



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ROTI®kit for High Throughput Screening

Introduction and product description:

The detection of cell proliferation is of utmost importance for assessing cell health, determining genotoxicity or evaluating anticancer drugs. This is normally performed by adding nucleoside analogs like [^{3}H]thymidine or 5-bromo-2'-deoxyuridine (BrdU) to cells during replication, and their incorporation into DNA is detected or visualized by autoradiography or with an anti-BrdU-antibody respectively. Both methods exhibit several limitations. Working with [^{3}H]thymidine is troublesome because of its radioactivity. Autoradiography is slow and thus not suitable for rapid high-throughput studies. The major disadvantage of BrdU staining is that the double-stranded DNA blocks the access of the anti-BrdU antibody to BrdU units. Therefore samples have to be subjected to harsh denaturing conditions resulting in degradation of the structure of the specimen.

Roth's *EdU Click HTS* assays overcome these limitations, providing a superior alternative to BrdU and [^{3}H]thymidine assays for directly measuring DNA synthesis of adherent cells in 96 well plates. EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. In contrast to BrdU assays, the *EdU Click HTS* assays are not antibody based and therefore do not require DNA denaturation for detection of the incorporated nucleoside. Instead, the *ROTI®kits for High Throughput Screening* utilize click chemistry for detection in a variety of dye fluorescent readouts. Furthermore, the streamlined detection protocol reduces both the total number of steps and significantly decreases the total amount of time. The simple click chemistry detection procedure is complete within 30 minutes and is compatible with multiplexing for content and context-rich results.

The *ROTI®kit for High Throughput Screening* can be used with antibodies against surface and intracellular markers. To ensure the compatibility of your reagent or antibody, please refer to **Table 1**.

Table 1: EdU detection dye compatibility

Fluorescent molecule	Compatibility
Organic dyes such as Fluorescein and Alexa dyes	Compatible
PerCP, Allophycocyanin (APC) and APC-based tandems	Compatible
R-phycoerythrin (R-PE) and R-PE based tandems	Use R-PE and R-PE based tandems **after** the EdU detection reaction
Quantum Dots	Use Quantum Dots **after** the EdU detection reaction
Fluorescent proteins (e.g. GFP)	Use anti-GFP antibodies* **before** the EdU detection reaction or use organic dye-based reagents for protein expression detection

* Compatibility indicates which involved components are unstable in the presence of copper catalyst for the EdU detection reaction (either the fluorescent dye itself or the detection method). Not all GFP antibodies recognize the same antigen site. Rabbit and chicken anti-GFP antibodies result in a good fluorescent amount. The mouse monoclonal antibodies tested are not recommended for this application because they do not generate an acceptable amount of fluorescence.

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Please read the material safety data sheets (MSDS) provided for this kit.

Cautions:

EdU (**Component A**): Danger H340-H360
P202-P280-P308+P313

Reaction Buffer (**Component C**): Warning H315-H319
P280-P302+P352a-P305+P351+P338

Catalyst Solution (**Component D**): Warning H302-H315-H319-H400-H410
P280-P301+P312a-P302+P352a-P305+P351+P338

Rinse Buffer (**Component F**): H412-EUH032
P273

The *rinse buffer* (**Component F**) is stored at RT and will crystallize at lower temperatures. In later case the solution has to be brought to RT, mixed thoroughly and can then, once homogenously dissolved, be used without further considerations. The activity of this compound is not affected hereby.

MSDS: the appropriate MSDS can be downloaded from our website www.carlroth.com.

Literature Citation: When describing a procedure for publication using this product, please refer to it as *Carl Roth's ROTI®kit for High Throughput Screening (EdU Click HTS)*.

1. Materials provided with the kit and storage conditions

Table 2: Contents of the kit and storage conditions

Vial-label	Amount for 2 assays/well plates	Amount for 4 assays/well plates	Amount for 20 assays/well plates	Component	Component long term storage	Kit storage*
Component A yellow	2 mL	4 mL	20 mL	5-Ethynyl-deoxyuridine (5-EdU)	-20 °C	2 – 8 °C Dark Do not freeze Dry
Component B red	130 µL	2 x 130 µL	9 x 130 µL	6-FAM-Azide 5-TAMRA-PEG3-Azide 5/6-Sulforhodamine-101-PEG3-Azide Eterneon Red 645-Azide	-20 °C dark	
Component C orange	20 mL	40 mL	4 x 50 mL	Reaction buffer	RT	
Component D green	1 mL	1 mL	5 mL	Catalyst solution	RT	
Component E blue	200 mg	400 mg	2 x 1 g	Buffer additive	-20 °C	
Component F grey	6 mL	2 x 6 mL	58 mL	Rinse buffer (10x)	RT	RT

*This kit is stable up to 1 year after receipt, when stored as directed.

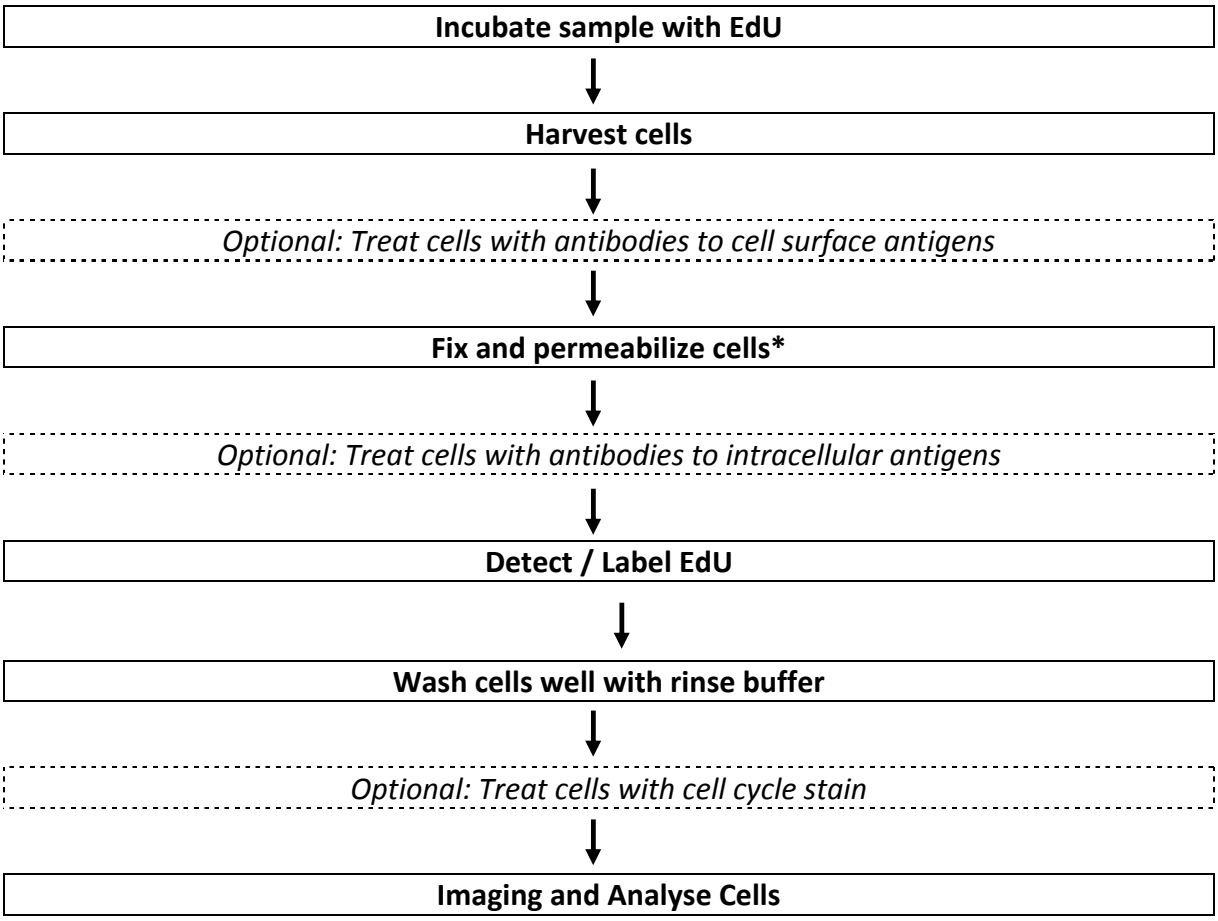
2. Required Material and Equipment not included in this Kit

- Adherent cells
- Reaction tubes (size depends on the volume of reaction cocktail needed)
- Buffered saline solution, such as PBS, DPBS or TBS
- Fixative solution (4% Paraformaldehyde in PBS)
- Saponin-based permeabilization and wash reagent (10x solution)
- Appropriate cell culture medium
- 1% BSA (bovine serum albumin) in PBS, pH 7.1 – 7.4
- 18 MΩ purified water

3. Workflow

The following protocol was developed using a final EdU concentration of 10 µM and can be adapted for any cell type. There are many factors which can influence the labeling such as the growth medium, the density and the type of cells. To determine the optimal concentration for your experiment, a range of EdU concentrations should be tested for your cell type and experimental conditions.
Principally, a similar concentration to BrdU can be used for EdU as a starting point. Heparin can be used as anticoagulant for collection, if a whole blood sample is used.

Workflow scheme for the *EdU Click HTS Assay*


Incubate sample with EdU
Harvest cells
Optional: Treat cells with antibodies to cell surface antigens
Fix and permeabilize cells*
Optional: Treat cells with antibodies to intracellular antigens
Detect / Label EdU
Wash cells well with rinse buffer
Optional: Treat cells with cell cycle stain
Imaging and Analyse Cells

* At this point the sample can be stored safely

4. Preparation of the stock solutions

4.1 Allow all vials to warm to room temperature before opening.

4.1.1 For the preparation of a 20 µM stock solution of EdU (2x EdU), add the appropriate amount of aqueous solution (1x PBS) to EdU (**Component A**) according to **table 3** and mix until the compound is completely dissolved. After use, store any remaining solution at -20°C. When stored as directed, this stock solution is stable for up to one year.

Table 3: Amounts of aqueous solution needed to dissolve EdU to a final concentration of 20 µM

EdU HTS Kit	20x EdU solution (Component A)	In dilution Volume for 2x EdU solution in PBS
1 well plate	1 mL	9 mL
2 x 96 well plates	2 mL	18 mL
4 x 96 well plates	4 mL	36 mL
10 x 96 well plates	10 mL	90 mL
20 x 96 well plates	20 mL	180 mL

4.1.2 For the preparation of a stock solution of the buffer additive, add the appropriate amount of deionized water (see table 4) to the **Component E** and mix until the compound is dissolved completely. After use, store any remaining solution at -20°C. When stored as directed, this stock solution is stable for up to 6 months. We recommend preparing aliquots to avoid repeated thaw and freeze cycles!

Table 4: Amounts of aqueous solution needed to dissolve the buffer additive to the final work solution

EdU HTS Kit	Buffer additive (solid) Component E	Dilution volume of deionized water
1 well plate	100 mg	1 mL
2 x 96 well plates	200 mg	2.5 mL
4 x 96 well plates	400 mg	5 mL
10 x 96 well plates	1 g	10 mL
20 x 96 well plates	2 g	25 mL

5. Labeling of cells with EdU

5.1 Suspend the cells in an appropriate tissue culture medium to obtain optimal cell growth conditions. Please note that the growth of the cells during incubation decelerates, if the temperature changes or the cells are washed prior to incubation with EdU.

5.2 For the desired final concentration, add the appropriate amount of EdU to the culture medium and mix well. We recommend using a concentration of 10 µM for 1-4 hours as a starting point. Use higher EdU concentrations for a shorter incubation time. A longer incubation time requires lower EdU concentrations.

5.3 The incubation of the cells with EdU should be performed under the optimal conditions for your cell type, the number of cells plated and for the desired length of time. Various DNA synthesis and proliferation parameters can be evaluated by altering the EdU incubation time or by subjecting the cells to pulse labeling with EdU. Effective time intervals for pulse labeling and the length of each pulse depend on the cell growth rate.

5.4 If performing antibody surface labeling, proceed immediately to step **6**, otherwise continue to step **7**.

6. Staining cell-surface antigens with antibodies (optional)

6.1 Wash cells in each well with 100 µL of 1% BSA in PBS.

6.2 Remove the wash solution and add again 100 µL of 1% BSA in PBS to the cells.

6.3 Add surface antibodies and mix well but gently.

Note: PE, PE-tandem or Quantum Dot antibody conjugates should not be used before performing the click reaction (step **8**).

6.5 Incubate the cells for the recommended length of time and temperature. Protect from light!

6.6 Proceed to step **7**.

7. Cell fixation and permeabilization

This protocol was developed with a fixation step using 4% Paraformaldehyde in PBS, followed by a saponin-based permeabilization step. The saponin-based permeablization and wash reagent can be used with cell samples containing red blood cells or whole blood as well as with cell probes containing different cell types. The morphological light scatter characteristics of leukocytes are maintained by the permeabilization reagent while red blood cells are lysed.

7.1 Remove the incubation media and wash the cells, each well with 100 µL of 1% BSA in PBS. Afterwards remove the wash solution.

7.2 Add 100 µL of the fixative solution to the cells in each well. Incubate for 15 minutes at room temperature. Protect from light.

7.3 Remove the fixation solution and wash the cells in each well twice with 200 µL of 1% BSA in PBS. If red blood cells or haemoglobin are present in the sample, repeat the washing step. Remove all residual blood cell debris and haemoglobin before proceeding.

NOTE: At this point of the procedure the probes can be stored safely.

7.4 Remove the wash solution and add to each well 100 µL of 1x saponin-based permeabilization buffer in PBS. Mix well but gently and proceed to step **8.** for the click reaction.

8. EdU detection

8.1 Prepare the click assay cocktail in the same order as described in **table 5**. If the ingredients are not added in the order listed, the reaction will not proceed optimally or might even fail.

Important: Once the assay cocktail is prepared, use it immediately, at least within the next 15 minutes!

Table 5: Click assay cocktails

Material	Component	Number of well plates				
		1	2	4	10	20
Reaction buffer	**C- orange**	9.64 mL	19.27 mL	38.54 mL	96.35 mL	192.7 mL
Catalyst solution	**D - green**	220 µL	440 µL	880 µL	2.2 mL	4.4 mL
Dye Azide (10 mM)	**B - red**	55 µL	110 µL	220 µL	550 µL	1.1 mL
Buffer additive (prepared in **4.1.2**)	**E - blue**	1.1 mL	2.2 mL	4.4 mL	11 mL	22 mL
Total Volume	-	**11.01 mL**	**22.02 mL**	**44.04 mL**	**110.1 mL**	**220.2 mL**

8.2 Remove Wash solution from step 7.4 and add 100 µL of the click assay cocktail to each well and mix well but gently to distribute the assay solution evenly.

8.3 Incubate the click assay mixture for 30 minutes at room temperature. Protect from light!

8.4 From the 10x rinse solution prepare a 1x rinse solution by applying following table (table 6). Add the appropriate amount of PBS (1x) (see **table 6**) to the **Component F** and mix well. This additional wash step with this special rinse buffer reduces unspecific, cell number dependent background signal. After use, store any remaining solution at RT. When stored as directed, this stock solution is stable for up to 6 months.

Table 6: Amounts of aqueous solution needed to prepare the 1x rinse buffer

EdU HTS Kit	Volume of 10x rinse buffer (Component F)	Dilution volume of 1x PBS
1 well plate	2.9 mL	26.1 mL
2 x 96 well plate	5.8 mL	52.2 mL
4 x 96 well plate	11.5 mL	103.5 mL
10 x 96 well plate	28.8 mL	259.2 mL
20 x 96 well plate	57.6 mL	518.4 mL

Remove click assay cocktail and wash the cells in each well twice with 150 µL with the 1x rinse solution prepared above.

8.5 Remove rinse solution. 100 µL of 1% BSA in PBS is then given to the cells in each well.

8.6 If performing antibody surface or intracellular labeling, proceed immediately to step **9**, otherwise continue to step **10**.

9. Staining intracellular or surface antigens (optional)

9.1 Add antibodies against intracellular antigens or against surface antigens that use RPE, PR-tandem or Quantum Dot antibody conjugates. Mix well.

9.2 Incubate the cells for the time and temperature required for antibody staining. Protect from light.

9.3 Wash each well twice with 100 µL 1x saponin-based permeabilization and wash reagent. Remove the solution. Add again 100 µL of 1% BSA in PBS to the cells.

9.4 Proceed with step **10** for analyzing the cells.

10. Imaging and analysis

10.1 Close the 96 well plate by using a sealing film, if desired.

10.2 Fluorescence is quantified by scanning the plate using an automated imaging platform equipped with filters appropriate for the dye used. Images of each well can be taken by microscopy.

The Excitation and emission maxima of the available dyes are listed in **table 7**.

Table 7: Emission and excitation maxima of the available dyes.

Product number	Dye	Excitation (nm)	Emission (nm)	Filter
7786.1 7787.1 7788.1	6-FAM-Azide	496	516	Green
7789.1 7791.1 7792.1	5-TAMRA-PEG3-Azide	546	579	Violet
7793.1 7794.1 7795.1	5/6-Sulforhodamine 101-PEG3-Azide	584	603	Orange
7796.1 7797.1 7798.1	Eterneon Red 645-Azide (Cyanine 5 Azide analogue)	643	662	Red

11. Example of the data derived from an EdU Click HTS Kit based experiment:

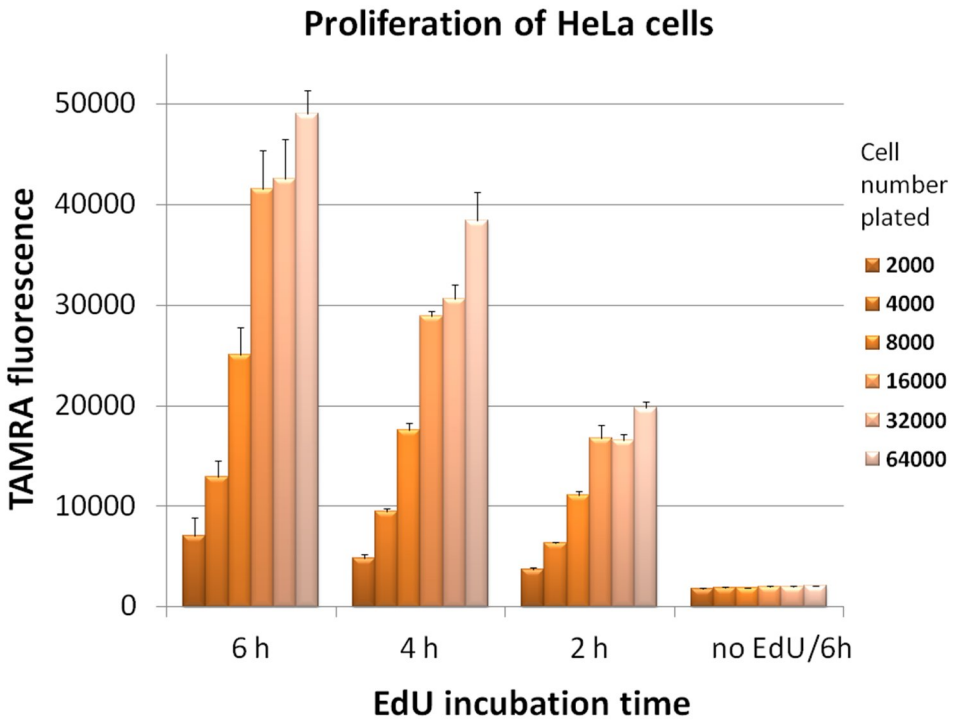


Figure 1. Detection of EdU incorporation depending on cell number and EdU incubation time.
HeLa cells were seeded in a transparent 96 well cell culture plate with indicated cell numbers per well. After 42 h cells were incubated with or without 10 µM EdU for 2, 4 or 6 h and subsequently EdU incorporation was detected using the EdU Click HTS-555 Assay Kit and a fluorescence plate reader. Mean and SD values from quadruplicates are shown.

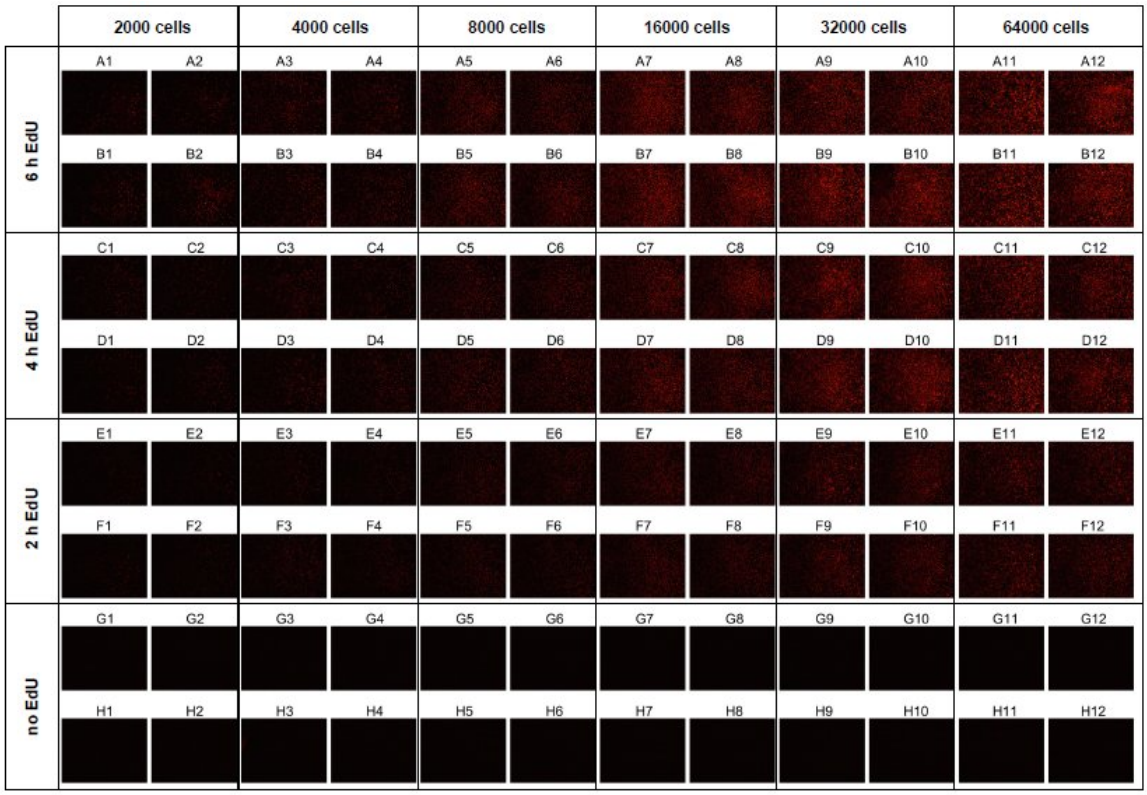


Figure 2: Detection of EdU incorporation via fluorescence microscopy.
A fluorescence photograph (40x) of the center of each 96 well of the, with rinse buffer washed assay plate was captured and presented using the Nikon NIS-elements software.

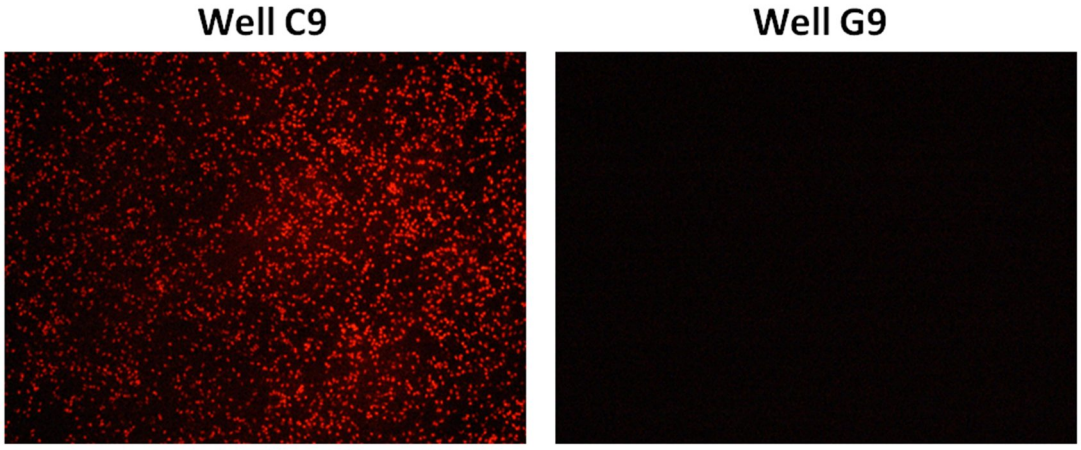


Figure 3: Zoom on the samples after Click reaction and washing (in Figure 5) cells which do EdU proliferation in well C9 and cells, which haven't received EdU, in well G9.

***Your notes*:**

***Your notes*:**

Ordering information:

(for detailed kit content see Table 2)

ROTI®kits for High Throughput Screening (for 2 x 96 well plate assays):

Product number	Product	Used fluorescent dye
7786.1	EdU Click HTS 2-488	6-FAM-Azide
7789.1	EdU Click HTS 2-555	5-TAMRA-PEG3-Azide
7793.1	EdU Click HTS 2-594	5/6-Sulforhodamine 101-PEG3-Azide
7796.1	EdU Click HTS 2-647	Eterneon-Red 645 Azide (Cyanine 5 Azide analogue)

ROTI®kits for High Throughput Screening (for 4 x 96 well plate assays):

Product number	Product	Used fluorescent dye
7787.1	EdU Click HTS 4-488	6-FAM-Azide
7791.1	EdU Click HTS 4-555	5-TAMRA-PEG3-Azide
7794.1	EdU Click HTS 4-594	5/6-Sulforhodamine 101-PEG3-Azide
7797.1	EdU Click HTS 4-647	Eterneon-Red 645 Azide (Cyanine 5 Azide analogue)

ROTI®kits for High Throughput Screening (for 20 x 96 well plate assays):

Product number	Product	Used fluorescent dye
7788.1	EdU Click HTS 20-488	6-FAM-Azide
7792.1	EdU Click HTS 20-555	5-TAMRA-PEG3-Azide
7795.1	EdU Click HTS 20-594	5/6-Sulforhodamine 101-PEG3-Azide
7798.1	EdU Click HTS 20-647	Eterneon-Red 645 Azide (Cyanine 5 Azide analogue)

To place your order, please contact us under:

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